Persistence of *Mycobacterium avium subsp. paratuberculosis* and other Pathogens during Composting, Manure Pack and Liquid Storage of Dairy Manure

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ABSTRACT. Manure and composts are important sources of agricultural nutrients. However livestock manures contain numerous pathogens which can infect humans and/or animals such as *Salmonella spp., Escherichia coli O157:H7, Listeria monocytogenes* and *Mycobacterium avium subsp. paratuberculosis (MAP),* the causative agent of Johne's disease. The objective of this study was to compare the persistence of naturally occurring and artificially inoculated MAP as well as other naturally occurring pathogens during the treatment of dairy manure by three different commonly used methods; thermophilic composting (55 C.), manure packing and liquid storage. A comparison was also done between straw and sawdust amendments used for composting and packing. Manure was obtained from the alleyway of a large Ohio free stall dairy farm that naturally contained the species described above. The manure was further inoculated with 10^6 CFU MAP. For compost and pack experiments, 6.5 kg of the manure was amended with 1.5 kg of sawdust or straw to give a moisture content of 60% ideal for composting or packing. One kg aliquots of these mixes were added to 4-liter compost reactors and incubated at 25 or 55 C in triplicate. For liquid storage, 2.6 kg of water was added to 10.3 kg of manure (to simulate liquid flushing and storage) and placed in 4-liter Erlenmeyer flasks in triplicate. The treatments were sampled on days 0, 3, 7, 14, 28 and 56 and analyzed for the presence of the pathogens described above by standard methods and for MAP by PCR product hybridization assay based on the MAP specific IS 900 integration site. Detection levels were less than 20 cells/gm. Chemical, physical and biological properties of the samples were also determined. Results indicated that *E.coli, Salmonella,* and *Listeria* were not detectable after 3 days of thermophilic composting but that they persisted up to 28 days in liquid manure and sawdust pack. MAP was detected by standard culture only on day 0 in the compost and pack treatments, but was detectable through day 28 in the liquid storage treatment. However, MAP DNA was detected through day 56 in all treatments. A quantitative analysis is underway to define MAP loads in each treatment.

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Introduction

Manures and composts are important sources of agricultural nutrients as organic amendments. Due to odor and handling problems, manure is often composted before field application. Composting continues to gain importance as a method of conversion of organic by-products (de Bertoldi et al., 1996). It is a biological process in which organic materials decompose mainly in an aerobic environment to release heat, water and CO_2. During composting, bacteria, fungi, and other microorganisms, including microarthropods, break down organic materials to stable, usable, organic substances called composts. Composting systems vary from static to aerated piles/windrows in the open environment or can be in-vessel or tunnels depending upon the material to be composted based on environmental issues and economics (Keener et al. 2000). Sludge and manures are generally composted as static or aerated piles/windrows and there are concerns about the potential contamination of agricultural produce with animal pathogens present in the compost if the process is not controlled properly (Keener et al. 2000).

Livestock manure contains numerous pathogens which can infect humans such as *Salmonella* sp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Cryptosporidium parvum* and *Giardia* sp. (Pell, 1997). *M. avium* subspecies *paratuberculosis* (*M. paratuberculosis)* is the causal organism of Paratuberculosis or Johne's disease which is a common and chronic disease of the intestines. The disease occurs worldwide among cattle, and has also been reported in other domesticated ruminants, wild ruminants as well as non-ruminant species. Estimated economic losses of millions of dollars have been reported annually due to animal culling, lowered milk production, reduced carcass value and poor reproductive performance (Ott, 1999). Since MAP bacteria are shed in feces as well, disease can be spread through contaminated foods, water or objects. As there are no vaccines to protect against infection, it is important to control spread of this pathogen through manure management and other prophylactic methods. *M. paratuberculosis* is also important due to its possible link with Crohn's disease (chronic inflammatory bowel) in humans (El-Zaatari et al. 2001).

Survival of microorganisms during manure treatment depends upon factors such as storage temperature, compost amendments, moisture content, redox potential, pH, physical composition and inter microbial competition (Turner, 2002). There are only a few studies on the survival of *E. coli*, *Salmonella*, and other organisms during composting of manure (Tiquia, 1998; Turner, 2002) and sludge mixed with Municipal waste (Krogstad & Gudding 1975). Himathongkham et al. 1999 used a culture technique to study the survival of *E. coli* O157:H7 and *S. typhimurium* in cattle manure and slurry stored at 4, 20, and 37°C. They observed an exponential linear destruction for *E. coli* O157:H7 and *S. typhimurium* in which decimal reduction times ranged from 6 days to 3 weeks in manure and from 2 days to 5 weeks in manure slurry. However, there is no research report on the survival of MAP in composted animal wastes (Collins, 2003).

To monitor the presence of MAP, a culture based detection method is generally used. However, since MAP is extremely slow growing (8-16 weeks) on artificial medium, despite decontamination steps, cultures are often lost because of overgrowth by contaminants. Furthermore, even the most sensitive culture techniques have only 50% sensitivity (Stabel et al. 2002). ELISA and other serologic tests such as agar gel immunodiffusion (Ferreira et al. 2002) and complement fixation (Kalis et al. 2002) have low specificity and sensitivity. Detection of MAP-specific nucleic acids has been recommended as quick indicator of the target bacteria

(Vary et al 1990). Further, specificity of the PCR amplification is confirmed by using specific probes in microtiter plate hybridization assays (Sreevatsan et al.2000). Although PCR cannot distinguish between live and dead organisms, it is frequently used as a detection tool.

The hypothesis for this research was that heat and microbial activity produced during the composting of manure can kill/eliminate animal pathogens as compared to the same manure stored as a liquid in a lagoon system. Therefore, objectives for this study were to assess persistence of naturally occurring and artificially inoculated (spiked) MAP, and persistence of other naturally occurring pathogens including *Salmonella, E. coli,* and *Listeria* during composting and simulated lagoon storage of manure. Also determined were the effects of temperature during composting on the survival of these pathogens. The third objective was to determine the effects of straw and sawdust compost amendments on the survival of pathogens. To study these objectives we used culture and PCR techniques to monitor the presence of MAP and culture methods to assess the presence of other pathogenic and indicator organisms. Changes in the physicochemical properties of the compost and lagoon stored materials were also monitored during the process to correlate with microbiological changes.

Materials and methods

Treatments and experiment set up. A laboratory experiment was set up to study the survival of the animal pathogens during composting and lagoon storage with the following 5 treatments. Three replications of each treatment were used:

1. Sawdust amended manure compost at 55°C
2. Straw amended manure compost at 55°C
3. Sawdust amended manure compost at 25°C
4. Straw amended manure compost at 25°C
5. Lagoon at room temperature.

Composting for the first four treatments was performed using a bench scale compost reactor system following Michel and Reddy (1998) with some modifications (Fig. 1). The system consisted of 4 liter capacity vessels (length 30 cm and diameter 15cm), made of PVC pipe, placed in an incubator (BioCold Environmental, Inc. Fenton, MO) set at 55°C and two incubators (Sheldon manufacturing Inc. Cornelius, OR) set at 25°C. Compost mixes in the vessels were placed on two metal screens of 1cm and 1mm mesh sizes, respectively. To achieve aerobic composting process for these treatments, regulated air at 31psi and -40°C dew point was blown through 0.6 cm diameter plastic tubes at 100 ml/min flow rate. Air was bubbled through a bottle containing water to humidify air entering the compost vessel to avoid moisture loss during composting. Moist air from the bottle entered at the bottom of the vessel via plastic tube attached to air inlet. Air was exhausted at the top of the vessel through plastic tube attached to air outlet and bubbled through flasks containing 100 ml of fresh 0.67 M boric acid solution with methyl red/bromocresol green indicators to trap ammonia. These flasks were placed in water bath set at 9°C to condense moisture from the air and to regulate the temperature of the boric acid solution. Air collected from flasks via plastic tubes was analyzed for CO_2 concentration using three of Vaisala model GMT 220 (range 0 to 20%) and O_2 concentration using three of Mine Safety appliances model "ULTIMA" (range 0-25%). Instrumentation system included a 3-way valve sequencer to switch the gas sampling lines from one set of vessels to the next. Data

was automatically recorded in Campbell Scientific model 23XL data logger every 12 minutes. Further, each vessel was equipped with a K type thermocouple to measure temperature in the mix near middle of the compost and data was recorded automatically every 12 minutes in the data loggers. A separate sensor of Vaisala model HMP 235 was used to monitor room conditions in the range of 0-100% relative humidity and -29 to 82°C temperature. Data stored on tape in the data loggers was transferred to a computer and processed with programs written for this purpose.

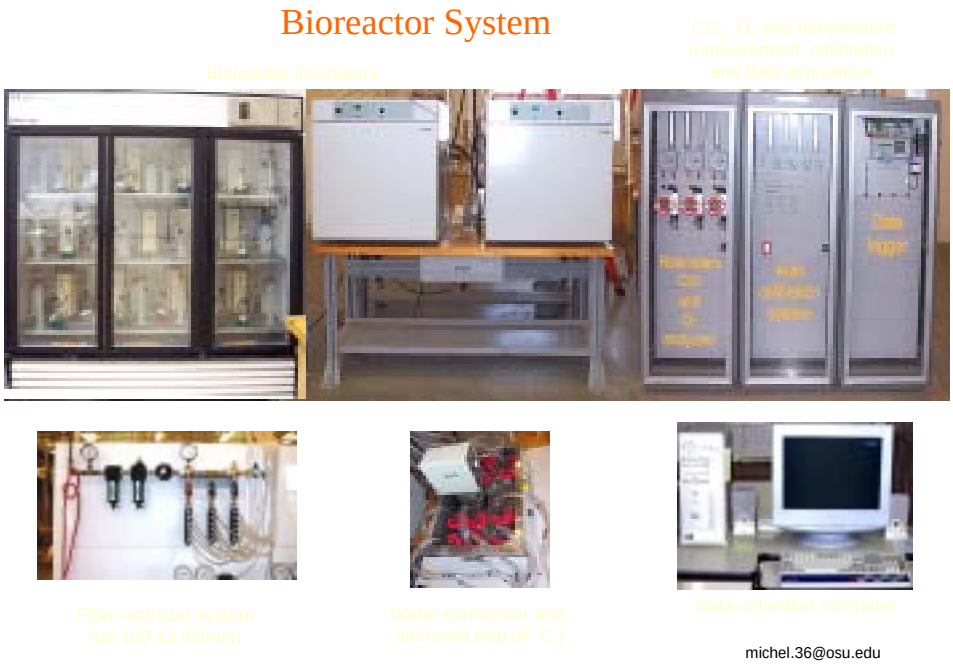


Figure 1. Compost reactor system used for pathogen survival studies

Lagoon treatment was simulated with diluted fecal drag stored undisturbed in 4 liter flasks at room temperature. The flasks were loosely covered with paper towels. Manure fecal drag with sand bedding was collected from a >2000 cow diary farm in Wayne county Ohio. Moisture content of manure fecal drag, sawdust and straw was 73.28, 5.53 and 6.53%, respectively. For sawdust and straw compost treatments, 6.5 kg of manure fecal drag was mixed with 1.5 kg of sawdust or straw to achieve desired moisture content of approximately 60%. About 2.6 liter of water was added to 10.3 kg of manure fecal drag to increase the moisture content to about 90% for lagoon treatment.

A suspension was prepared in PBS media, from 3 sheep and 2 human isolates/strains of *Mycobacterium avium* subsp. *paratuberoculosis* growing on media slants. Concentration of bacterial cells was determined on the basis of optical density at 600 nm. MAP suspension was mixed in the manure fecal drag used for each treatment. Compost mixes (with 10^6 cfu/g) were made using MAP spiked manure and sawdust or straw amendments. On wet weight basis, 1.115 kg, 1.025 kg and 4 kg of mixes were added to each composter or flask for sawdust, straw and lagoon treatments, respectively. Vessels and flasks were weighed before and after filling with mixes.

Physico-chemical analyses of composting material. Mixes in the above five treatments were composted for 56 days. Vessels and flasks were weighed before and after taking samples at each sampling interval to calculate weight loss during composting. Height of the vessel with compost mix was recorded to calculate density of each mix. Vessel contents were remixed before taking samples in a plastic bucket at each sampling date. Samples (approximately 100 g wet weight) were collected at 0, 3, 7, 14, 28, and 56 days after the start of the experiment for the assessment of animal pathogens and were stored at 4°C until further processed. Approximately 10 g of sample were dried in porcelain cups at 70°C for 24 h to check moisture in the mix at each sampling date. Samples at day 0 and 56 were also collected and submitted to a soil testing and research laboratory, OARDC, Wooster, Ohio to determine initial and final pH, organic matter, ash, nitrogen, and carbon content. Temperatures inside the vessels as well as room temperature, CO_2 and O_2 concentrations were recorded every twelve minutes.

Microbiological analyses of composting material

*Assessment of MAP survival by PCR methods***.** All samples were processed to check the presence of MAP. DNA was extracted from thoroughly mixed samples following Ozbek et al., 2003 with slight modifications.

To check the presence of M. *avium* subsp. *paratuberoculosis*, extracted DNA was amplified for *IS* 900 using MPARA 2 (5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3') as forward (Millar et al. 1996) and MPARA 1 (5'-/5'bio/GAG GTC GAT CGC CCA CGT GAC-3') as reverse (Reference) primer, respectively. Each PCR reaction mixture contained 10 µl of DNA extract, 9.67 µl of sterile distilled water, 1.5 µl of Dimethyl sulphoxide (Mallinckrodt cat. # 5507) spectrophotometric grade, 3 µl of 10X buffer (Promega, Corp., Madison, Wis.), 2.4 µl of 25 mM $MgCl_2$, 0.48 µl of 10 mg/ml bovine serum albumin (BSA), 0.60 µl of 10 µM of each primer, 0.75 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 µl of HotStar *taq* DNA polymerase (5 units/µl) in a final volume of 30 µl. The reaction mixture was incubated at 95°C for 15 min, cycled 35 (at 94°C for 15 sec, at 58°C for 20 sec and at 72°C for 20 sec) and incubated for 7 min at 72°C in a PTC-200 thermal cycler (MJ Research Inc., Massachusetts, USA). A PCR blank was included for each batch. All PCR reactions were stored at 4°C and were used immediately for hybridization assay.

The biotinylated amplicons were detected by hybridizing the products to integration site-specific probe PRmpara (5'-GCG GGT GGC CAA CGA CGA GGC CGC GCT GCT GGA GTT GA-3') coated on a microtiter plate at 100 ng/ well as described by Sreevatsan et al. 2000. Manure negative for MAP, PCR blanks and hybridization negatives were used as negative controls in all batches. MAP suspension used for the experiment included as a positive control.

Results

Physico-chemical Changes. Results for physico-chemical properties of compost mixes at the start and end of the composting period are presented in Table 1. Compost mixes at the time of start of the experiment had 41.7 and 40% solids with pH 8.7 and 8.6 in sawdust and straw compost mix, respectively. Lagoon treatment had 78% moisture content with pH 7.8 at the start of the experiment. Ash content and % nitrogen were highest in lagoon treatments whereas carbon content was lowest. At the time of termination of the experiment, all the treatments had lower C:N ratios than the start. There was a slight decrease in pH of all the compost mixes.

Table 1. Initial and final physico-chemical properties of the compost mixes.

	pH	% Solids	% Volatile solids	% Total N	% Total C	C:N ratio
Initial						
Sawdust	8.7±0.3	41.7±0.6	58.6±0.8	0.7±0.1	28.7±0.4	42.3±5.1
Straw	8.6±0.1	40.0±0.9	51.2±3.6	0.9±0.1	25.7±1.3	30.4±1.7
Lagoon	7.8	19.08	25.9	1.01	12.95	12.1
Manure	8.0±0.03	27.2±1.4	25.9±1.4	1.4±0.15	14.6±1.05	10.5±0.4
Final						
Sawdust 55	7.8±0.0	36.6 ± 0.8	33.7±1.9	1.1±0.0	17.2±0.7	15.4±0.8
Straw 55	8.0±0.1	37.6±1.7	19.7±0.7	1.2±0.0	10.2±0.1	8.5±0.3
Sawdust 25	8.3±0.1	32.5±0.3	37.1±1.2	1.2±0.0	20.1±0.9	17.5±1.2
Straw 25	7.7±0.1	31.9±0.3	31.2±0.9	1.4±0.1	15.5±0.8	11.0±0.2
Lagoon	7.2±0.1	13.2±2.8	39.3±10.8	2±0.6	21.3±5.4	10.8±0.3

Values are average of three replications ± Standard deviation.

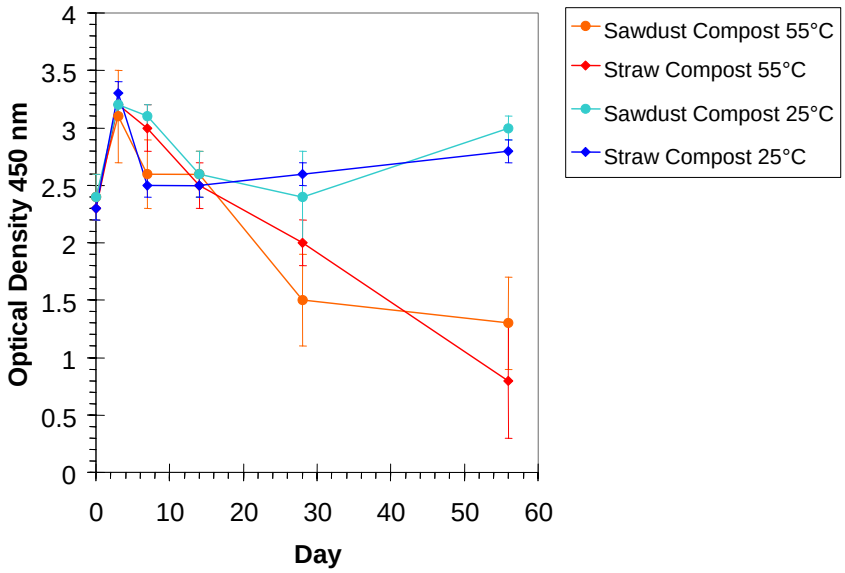
Temperatures of the composts and liquid manures were recorded by thermocouples every 12 minutes and saved in data loggers and daily means were calculated. There was an increase in temperature in both types of compost mixes incubated at 55°C as well as 25°C above the setpoint due to microbial activity. Maximum increase in temperature was on day 2 for compost mixes at 55 and on day 1 for compost mixes at 25°C. Straw amended manure mix showed an increase of 8°C at 55°C and 5°C at 25°C incubation where as sawdust amended manure had 4°C increase in temperature at both the incubations. Overall, temperature stayed 3-4°C high than the set up in all the mixes for about 10 days indicating high microbial activity during this time and temperature was 1-2°C high for the rest of the experimental duration (total 56 days).

Data recorded by O_2 analyzers, on % oxygen level in the exiting air from composters, showed that maximum oxygen consumption occurred during the first 2-3 days in all the treatments with heavy consumption in compost mixes incubated at 55°C. Straw amended compost mixes consumed more oxygen at both the temperatures as compared to sawdust compost mixes. Maximum decrease (15%) in % O_2 level was observed in straw treatment at 55°C. Higher CO_2 evolution was observed especially during first 2-3 days as indicated by the % CO_2 level in the exiting air recorded with CO_2 analyzers. Straw amended manure produced more CO_2 than sawdust manure mix especially at 55°C.

Microbiological changes

Persistence of MAP as determined by a PCR-hybridization assay. Results from PCR for the amplification of *IS* 900, and hybridization with specific probes, performed for each sample taken during composting process, are summarized in Figure 2. Data shows that all the samples taken during different intervals of composting were MAP DNA positive. Optical density was considerably lower in treatments at 55°C at day 28 and 56 sampling. Manure used for the experiment was also processed the same way and was found to be MAP positive although optical density was low (0.7-1.3) as compared to the inoculated treatments. MAP suspension sample positive controls showed an optical density of 3.0. Optical densities for manure negative for MAP, PCR blank and hybridization blank was below 0.2 (cut off value, Ozbek, et al. 2003; Motiwala, et al., 2004).

Figure 2. Survival of Mycobacterium avium paratuberculosis (Causative agent of Johnes Disease) by PCR product hybridization assay based on MAP specific IS 900 integration site.



Persistence of MAP as determined by a culture method. Examination of culture tubes at 8 weeks showed that all the treatments were MAP positive at the time of the start (day 0 samples) of the experiment. However, no sample was MAP culture positive at day 3 and 7. At day 14, one lagoon treatment replicate was found to be positive for the presence of MAP whereas all the three replications from the same treatment were positive at day 28. No MAP was detected from any other treatment replicates at days 14 and 28. At day 56, all the three replications of the lagoon treatment were positive for MAP whereas no MAP was detected in any of the other treatments.

Persistence of other microorganisms as determined by culture methods. Culture methods were used to check the survival of some of the other naturally occurring bacteria in manure and results are summarized in figure 3. *E. coli*, *Salmonella* spp. and *Listeria* sp. were present in manure as well as in all the treatments at day 0. However, at 55°C after 3 days none of these three bacteria were detected in either sawdust or straw amended composts. In addition, none were detected at day 56 of sampling in any treatment. At 25°C, *E. coli* was not detected in any sample of sawdust or straw manure at day 3 of sampling. However, it was present in 2 sawdust amended compost treatments and 3 of the straw amended compost replicates at day 7 and 14. *E. coli* was not detected at day 28 and 56 sampling of these treatments. In lagoon treatments at room temperature, it was present in all the three replications until day 14 and was not detected at day 28 and 56 (Fig. 3).

In 25°C compost, *Salmonella* spp. was only present in one sample of sawdust amended compost on day 3 and was not detected afterwards. Straw amended composts showed only one sample positive with *Salmonella* at day 7 and day 28. *Salmonella* survived at least for 7 days in all the replications of the lagoon treatment and only one sample was positive at day 14 and 28. *S. cerro* was present in all the samples at day 0 whereas *S. give* was also observed in some of the *Salmonella* positive samples on later dates. *Listeria* sp. was present in one sample of each of

sawdust, straw manure mix and lagoon treatments stored at 25°C, on day 3 whereas *Listeria* was not observed in any of the samples of any treatment by day 7. By day 14, *Listeria* was only present in 2 samples of the lagoon treatment. However, no *Listeria* was present on day 28 and 56 of sampling in any treatment.

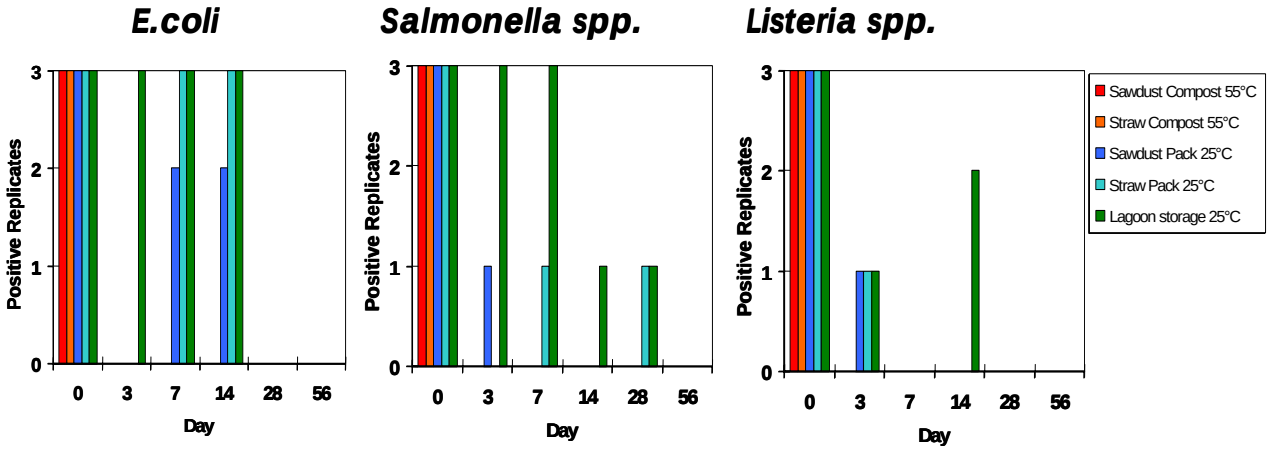


Figure 3. Persistence of pathogens during the composting and lagoon storage of dairy manure